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Report

Venezuelan equine encephalitis replicon immunization overcomes intrinsic tolerance and elicits effective anti-tumor immunity to the 'self' tumor-associated antigen, *neu* in a rat mammary tumor model

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Key words: breast cancer, immunotherapy, *neu*, rat tumor model, replicon vector

Summary

Many tumor-associated antigens (TAAs) represent 'self' antigens and as such, are subject to the constraints of immunologic tolerance. There are significant barriers to eliciting anti-tumor immune responses of sufficient magnitude. We have taken advantage of a Venezuelan equine encephalitis-derived alphavirus replicon vector system with documented *in vivo* tropism for immune system dendritic cells. We have overcome the intrinsic tolerance to the 'self' TAA rat *neu* and elicited an effective anti-tumor immune response using this alphavirus replicon vector system and a designed target antigen in a rigorous rat mammary tumor model. We have demonstrated the capacity to generate 50% protection in tumor challenge experiments ($p = 0.004$) and we have confirmed the establishment of immunologic memory by both second tumor challenge and Winn Assay ($p = 0.009$). Minor antibody responses were identified and supported the establishment of T helper type 1 (Th1) anti-tumor immune responses by isotype. Animals surviving in excess of 300 days with established effective anti-tumor immunity showed no signs of autoimmune phenomena. Together these experiments support the establishment of T lymphocyte dependent, Th1-biased anti-tumor immune responses to a non-mutated 'self' TAA in an aggressive tumor model. Importantly, this tumor model is subject to the constraints of immunologic tolerance present in animals with normal developmental, temporal, and anatomical expression of a non-mutated TAA. These data support the continued development and potential clinical application of this alphaviral replicon vector system and the use of appropriately designed target antigen sequences for anti-tumor immunotherapy.

Introduction

One in eight American women will be diagnosed with breast cancer [1]. Despite significant treatment advances, a substantial percentage of women diagnosed with breast cancer will develop metastatic disease, often after many years, suggesting the presence of micrometastatic disease after initial treatment. Novel treatment methods directed at seeking out and eliminating this persistent micrometastatic disease might have substantial clinical benefit. The immune system is particularly well suited for this purpose. Breast cancer patients have been documented to have immune responses to breast cancer tumor-associated antigens (TAAs) [2–8], but these immune responses are generally of low magnitude and are clearly insufficient to establish or maintain control of patients' tumors. Many TAAs have been characterized as 'altered self' [9] or are mal-expressed 'self' molecules, which may account, in part, for the difficulty encountered in attempts to elicit robust antigen-specific, anti-tumor immune responses due to intrinsic tolerance to 'self'. Recent promising studies in non-Hodgkins lymphoma and mela-

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noma have demonstrated the capacity of immunotherapeutic strategies to elicit TAA-specific immune responses that are associated with clinical responses [10–13]. However, the clinical benefit of anti-tumor immunotherapy in other solid tumors such as breast cancer remains to be demonstrated.

Alphaviruses, such as Venezuelan equine encephalitis (VEE), are positive strand ribonucleic acid (RNA) viruses that have several characteristics which are potentially advantageous for the derivation of anti-tumor immunotherapeutic/vaccine vector systems [14,15], including demonstrated tropism for a subset of immune system dendritic cells (DCs) [16]. Several strategies using vector systems derived from alpha viruses have been described, including viral replicon particles (VRPs) [17–30]. VRPs are single-cycle vectors containing RNA replicons with an engineered multiple cloning site in place of the viral structural protein genes. Heterologous genes cloned into this site are expressed from the 26S subgenomic RNA promoter at very high levels. Replicon particles are produced by providing the missing structural protein genes *in trans* on two helper RNAs [31]. The application of VEE VRPs for immunization with various infectious disease antigens (one of which is also a TAA) has been reported [27, 32–41]. The potency of VEE VRPs in these studies suggests that VRPs might have similar efficacy in targeting a 'self' TAA.

We have used a rat mammary tumor model and selected the *neu* molecule, homologue of human HER2/*neu*, as a prototypical TAA to test our hypothesis that the application of VEE derived VRP immunotherapy will overcome intrinsic tolerance and elicit efficacious anti-tumor immunity. The selection of a rat model is complicated by the availability of fewer immunologic reagents, but the normal expression pattern of rat *neu*, in contrast to mice transgenic for rat *neu*, provides for a model; (1) that more closely matches the human clinical situation, (2) with normal intrinsic tolerance, and (3) the potential to observe elicited autoimmune phenomenon.

Materials and methods

Animals and cell lines. Six- to eight-week-old Fisher 344 female rats (NCI-FCRC, Frederick, MD) were obtained and housed in grouped cages under normal vivarium conditions. Water and rodent chow were provided *ad libitum*. The rat mammary tumor cell line 13762 MAT B III (CRL-1666, ATCC, Manassas, VA) was obtained and cultured *in vitro* as recommended. Cells were harvested using Versene (Gibco Life technologies, Rockville, MD) and washed three times in phosphate-buffered saline prior to suspension in injection grade normal saline for inoculation into recipient animals. BHK cells (CCL-10, ATCC, Manassas, VA) were used for production and titration of VRPs. All work was performed under an approved and active animal experimental protocol. All experiments were performed with strict adherence to all institutional animal care and use guidelines. The expression level of rat *neu* and MHC class I molecules were routinely monitored by flow cytometry (FACScan, B.D. Biosciences, San Diego, CA) using FITC labeled appropriate isotype controls, Ab-4 (Oncogene Science, Boston, MA) and OX-18 (B.D. Biosciences, San Diego, CA) respectively, Figure 1.

Target antigen sequence. Partial protein sequences from HER2/*neu* were used to probe the protein databases for regions of homology to known proteins. Regions with the least amount of homology with other normal proteins, including other members of the epidermal growth factor receptor (EGFR) family, were selected for inclusion in the target antigen sequence. Regions from the extra-cellular domain were not included to optimize the likelihood of intracellular expression, MHC class I processing/presentation,

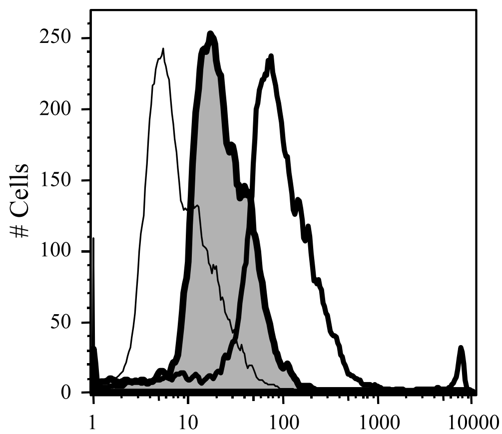

Figure 1. 13762 MAT B II expression of MHC class I and rat *neu*: Representative, non-gated, data from routine fluorescent cytometry monitoring of the 13762 MAT B II tumor line. MHC class I, bold line – no shading; rat *neu*, bold line – gray shading; and isotype control thin line – no shading.

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            v10       v20       v30       v40       v50       v60       v70       v80       v90       v100      v110      v120
   MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNG
   ||||| ||||:|||||||| |:||||||||||||||||||||||||||||||||||||||||||:|:||||||||||||||||:|||||||::|||||||||||||||||:||||||||
MIIMELAAWCRWGFLLALLPPGIAGTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYVPANASLSFLQDIQEVQGYMLIAHNQVKRVPLQRLRIVRGTQLFEDKYALAVLDNR
         ^10       ^20       ^30       ^40       ^50       ^60       ^70       ^80       ^90       ^100      ^110      ^120

         v130       v140      v150      v160     v170      v180      v190     v200      v210      v220      v230
DPLNNTTPVT-GASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQ
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DPQDNVAASTPGRTPEGLRELQLRSLTEILKGGVLIRGNPQLCYQDMVLWKDVFRKNNQLAPVDIDTNRSRACPPCAPACKDNHCWGESPEDCQILTGTICTSGCARCKGRLPTDCCHEQ
      ^130       ^140      ^150      ^160     ^170      ^180      ^190     ^200      ^210      ^220      ^230      ^240

v240      v250      v260      v270     v280      v290      v300      v310     v320      v330      v340      v350
CAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSA
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CAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMHNPEGRYTFGASCVTTCPYNYLSTEVGSCTLVCPPNNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLRGARAITSD
      ^250      ^260      ^270      ^280     ^290      ^300      ^310      ^320     ^330      ^340      ^350      ^360

v360      v370      v380      v390     v400      v410      v420      v430     v440      v450      v460      v470
NIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHT
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NVQEFDGCKKIFGSLAFLPESFDGDPSSGIAPLRPEQLQVFETLEEITGYLYISAWPDSLRDLSVFQNLRIIRGRILHDGAYSLTLQGLGIHSLGLRSLRELGSGLALIHRNAHLCFVHT
      ^370      ^380      ^390     ^400      ^410      ^420      ^430      ^440     ^450      ^460      ^470      ^480

v480      v490      v500       v510     v520      v530      v540      v550     v560      v570      v580      v590
VPWDQLFRNPHQALLHTANRPEDE-CVGEGLACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVA
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VPWDQLFRNPHQALLHSGNRPEEDLCVSSGLVCNSLCAHGHCWGPGPTQCVNCSHFLRGQECVEECRVWKGLPREYVSDKRCLPCHPECQPQNSSETCFGSEADQCAACAHYKDSSSCVA
      ^490      ^500      ^510     ^520      ^530      ^540      ^550      ^560     ^570      ^580      ^590      ^600

 v600      v610      v620      v630     v640      v650      v660      v670     v680      v690      v700      v710
RCPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIVSAVVGILLVVVLGVVFGILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKET
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RCPSGVKPDLSYMPIWKYPDEEGICQPCPINCTHSCVDLDERGCPAEQRASPVTFIIATVEGVLLFLILVVVVGILIKRRRQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKET
      ^610      ^620      ^630     ^640      ^650      ^660      ^670      ^680     ^690      ^700      ^710      ^720

 v720      v730      v740      v750     v760      v770      v780      v790     v800      v810      v820      v830
ELRKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLED
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ELRKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVREHRGRLGSQDLLNWCVQIAKGMSYLED
      ^730      ^740      ^750     ^760      ^770      ^780      ^790      ^800     ^810      ^820      ^830      ^840

 v840      v850      v860      v870     v880      v890      v900      v910     v920      v930      v940      v950
VRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKC
      ^850      ^860      ^870     ^880      ^890      ^900      ^910      ^920     ^930      ^940      ^950      ^960

 v960      v970      v980      v990     v1000     v1010     v1020     v1030    v1040     v1050     v1060     v1070
WMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGGDLTLGLEPSEEEAPRSPLAPS
||||||||||||||||||||||||||||||||||||||:||:||||||||||||||||||||||||||||||||.|||:||:|:.|:|||||||||||||:||||||||||.:||||||||
WMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPSSPMDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFSPDPTPGTGSTAHRRHRSSSTRSGGGELTLGLEPSEEGPPRSPLAPS
      ^970      ^980      ^990      ^1000    ^1010     ^1020     ^1030     ^1040    ^1050     ^1060     ^1070     ^1080

 v1080     v1090     v1100     v1110    v1120     v1130     v1140     v1150    v1160    v1170     v1180     v1190
EGAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERAKTLSPGKNGVVKDVFAFGGAVENPEYLT
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EGAGSDVFDGDLAMGVTKGLQSLSPHDLSPLQRYSEDPTLPLPPETDGYVAPLACSPQPEYVNQSEVQPQPPLTPEGPLPPVRPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEYLV
      ^1090     ^1100     ^1110     ^1120    ^1130     ^1140     ^1150     ^1160    ^1170     ^1180     ^1190     ^1200

 v1200     v1210     v1220     v1230    v1240     v1250
PQGGAAPQPHPPPAFSPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
|:.|:|:.|||:|||||||||||||||:::|:|:|||.|.|||||||||||||||||
PREGTASPPHPSPAFSPAFDNLYYWDQNSSEQGPPPSNFEGTPTAENPEYLGLDVPV
      ^1210     ^1220     ^1230     ^1240    ^1250      ^1260
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Figure 2. Comparative predicted amino acid sequences of human and rat c-erb B2. Standard single letter amino acid notations are used for the human sequence above and rat sequence below. Vertical bars = identical amino acids, colon = conservative substitutions, and periods = semi-conservative substitutions. Underlined regions represent those selected for inclusion in the target antigen sequence.

and T helper type 1 (Th1) immune responses. The exceptional degree of homology between HER2/*neu* and rat *neu* allowed the selection and construction of an entirely homologous target antigen sequence for rat *neu*, Figure 2. The selected sequences were obtained by rtPCR from mRNA isolated from the 13762 MAT B III tumor cell line. Total RNA was isolated using Trizol reagent and first strand cDNA synthesis was performed using Superscript II Reverse Transcriptase (Gibco Life technologies, Rockville, MD) according to manufacturers instructions. Pfu thermostable polymerase (Stratagene, LaJolla, CA) was used for all PCR reactions. The following primers were used to amplify a small fragment that includes the transmembrane domain, + strand (bases 1855–1882) 5′-CTCCTACATGCCCATCTGGAAGTACCC-3′ and − strand (bases 2093–2120) 5′-TAACTCAGTTTCCT GCAGCAGCCTACG-3′. The following primers were

used to amplify a larger fragment derived from the cytoplasmic domain, + strand (modified from bases 3110–3142) 5′-GATTCTTCTC***TCCGGAG***CCCTAC CCCAGGCAC-3′ and − strand (bases 3920–3950) 5′-CAGCAAGGAAAGGTTCCTCGGGGCAGGTTC-3′. The proximal fragment contains an intrinsic BspE1 site and the terminal fragment positive strand primer was modified by changing the 11th base from a C to T creating a BspE I site (underlined) and inserting a G between bases 16 and 17 in order to maintain the reading frame. These two fragments were digested with BspE I (New England Biolabs, Beverly, MA) under standard conditions, purified by agarose gel electrophoresis, and ligated using Rapid Ligation Kit (Gene Choice, PGC Scientifics Corp. Frederick, MD) as per manufacturers instructions. The resultant ligation product was used for PCR amplification of the target antigen sequence using the following primers containing engineered Bgl I sites for directional cloning (underline), + strand 5′-CCCATCGCCACCATGGCCTCCTGTGTGGATCT GGATGAACGAGGC-3′ and − strand 5′-ACGTGC CCTTAAGGCTCATACAGGTACATCCAGGCCTAG GTACTC-3′. The resulting target antigen sequence, encoding a heterologous target antigen protein containing 275 amino acids, incorporates a 'start' methionine (M) and alanine (A) in the second position preceding the sequence from the smaller fragment. The fusion of the polypeptides encoded by the smaller and larger PCR fragments required a conserved substitution of arginine (R) for lysine (K) and the insertion of serine (S) at the fusion site in order to maintain the reading frame through the BspE I site in the resultant fusion protein, Figure 3. This amplified tumor antigen sequence was sub-cloned into a standard cloning vector for sequence confirmation and subsequent sub-cloning into the VEE VRP production constructs and for production of His tagged target antigen protein.

MASCVDLDERGCPAEQRASPVTF*IIATVVGVLLF LILVVVVGILI*KRRRQKIRSPTPGTGSTAHRRHRS SSTRSGGGELTLGLEPSEEGPPRSPLAPSEGAGS DVFDGDLAMGVTKGLQSLSPHDLSPLQRYSED PTLPLPPETDGYVAPLACSPQPEYVNQSEVQPQ PPLTPEGPLPPVRPAGATLERPKTLSPGKNGVV KDVFAFGGAVENPEYLVPREGTASPPHPSPAFS PAFDNLYYWDQNSSEQGPPPSNFEGTPTAENP EYLGLDVPV

Figure 3. Amino acid sequence of the designed tumor-associated target antigen. Standard single letter amino acid notations are used. Underlined amino acids note modifications/additions to the wild type sequences, see text for full description, italicized sequence represents the transmembrane domain.

VEE VRP Production. The target antigen sequence, designed and constructed as above, and the influenza A hemagglutinin (H1) sequence were subcloned into the VEE replicon plasmid pVR200 that has been described previously [41]. This plasmid along with the two other plasmids encoding the structural gene sequences, the split helper plasmid system, were linearized by Not I digestion. *In vitro* transcription using T7 RNA polymerase was used to generate capped RNAs that were electroporated into BHK-21 cells for the production of the VRPs. VRPs were concentrated from culture supernatants via centrifugation through a 20% sucrose cushion prepared in Phosphate Buffered Saline (PBS). Infectious unit titers (IU/ml) were obtained by plating serial dilutions on BHK-21 monolayers with immunofluorescent evaluation of VEE non-structural gene products or heterologous protein expression. Extensive safety testing was performed prior to release of VEE VRPs for experimental evaluation to document the absence of replication competent virus. VRPs were resuspended in PBS with 1% normal rat serum and frozen at −80°C for shipment and diluted in this same buffer to an appropriate concentration for administration.

Immunization and phlebotomy. Animals were immunized with 200 μl of solution containing the appropriate concentration of VEE VRPs administered through a 27 g needle. All injections were performed with minimal restraining of conscious animals. The administration site was cleansed with 70% ethanol and allowed to dry prior to immunization. Subcutaneous (SC) immunizations were located approximately 0.5–1.0 cm cephalad and lateral to the base of the tail on the contralateral side to tumor inoculation. Intramuscular (IM) administrations were located in the quadriceps, rectus medius. Ten to 12 days after completion of immunization sequences, venous blood was obtained via standard saphenous vein phlebotomy. Serum was stored at −20°C prior to analysis.

*His-tagged r*Neu *protein expression and purification.* A histidine tag was added to the C-terminus of the rat *neu* target antigen coding sequence by PCR amplification using the following forward and reverse primers engineered to contain *Eco*RV and *Asc*I sites, respectively (sites underlined), 5′-CGGATATCATGGCCTCCTGTGTGGATCTG-3′ and

5′-TTGGCGCGCCTCAATGGTGATGGTGATGGTG TACAGGTACATCCAGGCCTA-3′. The PCR amplified product was digested with *Eco*RV and *Asc*I and ligated into a similarly digested alphaviral replicon vector (pERK) [39].

BHK cells were electroporated with RNA generated from the replicon-r*Neu*/His construct as described in [41]. Sixteen hours post-electroporation cells were washed with PBS and lysed in NP-40 lysis buffer (1% NP40, 50 mM NaPi pH 7.4, 0.3 M NaCl, 10 mM DOC, 20 mM Imidazole and mixture of protease inhibitors (Roche, Indianapolis, IN)). The lysates were cleared by centrifugation at 3000 RPM for 15 min at 4°C and subsequently filtered through a 5 μM Millex SV filter (Millipore, Billerica, MA). The r*Neu*/His protein was purified from the clarified lysates using Ni-NTA Superflow Columns (Qiagen, Valencia, CA) following the manufacturer's procedure.

ELISA. Nunc-Immuno MaxiSorb plates (Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 75 ng of r*Neu*/His protein/well diluted in carbonate–bicarbonate buffer pH 9.6 (Sigma, St. Louis, MO). The plates were then blocked with 3% BSA (Sigma) in PBS for 1 h at 30°C and then washed six times with PBS. Serial dilutions of sera from experimental animals, diluted in 1 × PBS with 1% BSA and 0.05% Tween 20 (Sigma), were plated in triplicate (50 μl/well) and incubated for 1 h at 30°C. Plates were washed six times with PBS and incubated 1 h with a 1:2000 dilution of HRP, Goat Anti-Rat, IGG (H + L) (KPL, Gaithersburg, MD). After washing as above, 100 μl of peroxidase substrate (ABTS Microwell Peroxidase Substrate System, KPL) was added to each well and plates were read at OD_{405} on a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA).

Isotyping. Sera from experimental animals that had detectable anti-r*Neu* target antigen antibody were isotyped for IgG1, IgG2a and IgG2b responses. Nunc-Immuno MaxiSorb plates (Nalge Nunc International) were coated overnight at 4°C with 75 ng of r*Neu*/His protein/well or with purified IgG1, IgG2a or IgG2b (Southern Biotech, Birmingham, Alabama) for standard curves from 500 ng/well to 3.9 ng/well diluted in carbonate–bicarbonate buffer, pH 9.6. Plates were blocked and washed as described above and subsequently incubated with rat sera diluted from 1:40 to 1:5120 for 1 h at 30°C. The plates were washed as above and then incubated with either a 1:250 dilution of goat anti-rat IgG1-AP, a 1:100 dilution of goat anti-rat IgG2a-AP, or a 1:75 dilution of goat anti-rat IgG2b-AP (Bethyl Laboratories, Montgomery, TX). Serum from each animal was tested in duplicate with each of the secondary antibodies. In addition, wells that were coated with dilutions of purified IgG1, IgG2a or IgG2b were incubated with the respective secondary antibody, conditions were established in which there was no cross-reactivity. Plates were washed, developed and read as described above.

Tumor challenge. Animals were immunized at 3-week intervals for a total of three immunizations with the appropriate dose and routes of administration for each cohort ($n = 6$). Tumor cells, 1×10^5 viable cells (preparations were >95% viable for use), were administered into the SC space on the flank, located 1 cm cephalad and lateral to the base of the tail. Tumors in control animals developed in 12–14 days. Tumor volumes were assessed and calculated using the formula, volume $= 0.4(ab^2)$ where 'a' and 'b' represent perpendicular axis measurements with 'a' representing the longest axis dimension [42]. Tumors that exceeded 10 cc or any signs of distress in the animals were indications for euthanization that was performed by CO_2 inhalation. Repeat tumor challenge, an equivalent number of cells, was administered to surviving animals on day 160 from initial tumor challenge, and placed contralateral to the original tumor challenge.

Winn assay. Animals ($n = 10$) received three immunizations as above with 1×10^7 IU VRPs. Two weeks after completing the immunization series, animals were euthanized and spleens were harvested. T cell enriched splenocytes were obtained by dissociation of the spleen and passage of cellular material through steel mesh, erythrocyte lysis (ACK buffer, Biosource International, Camarillo, CA), and passage over previously prepared autoclaved nylon wool columns (7.5 g of nylon wool (Robbins Scientific Corp., Sunnyvale, CA) packed into the barrel of a 60 cc syringe). Columns were incubated with RPMI 1640 supplemented with 10% FCS at 37°C for 45 min prior to loading of the washed splenocytes. After incubation at 37°C for 30 min the non-adherent cellular fraction was eluted off the column with 2× column volumes of RPMI-1640 supplemented with 10% FCS, collected and re-suspended in PBS. Appropriate numbers of immune T cell enriched splenocytes were added to a suspension of 13762 MAT B III mammary

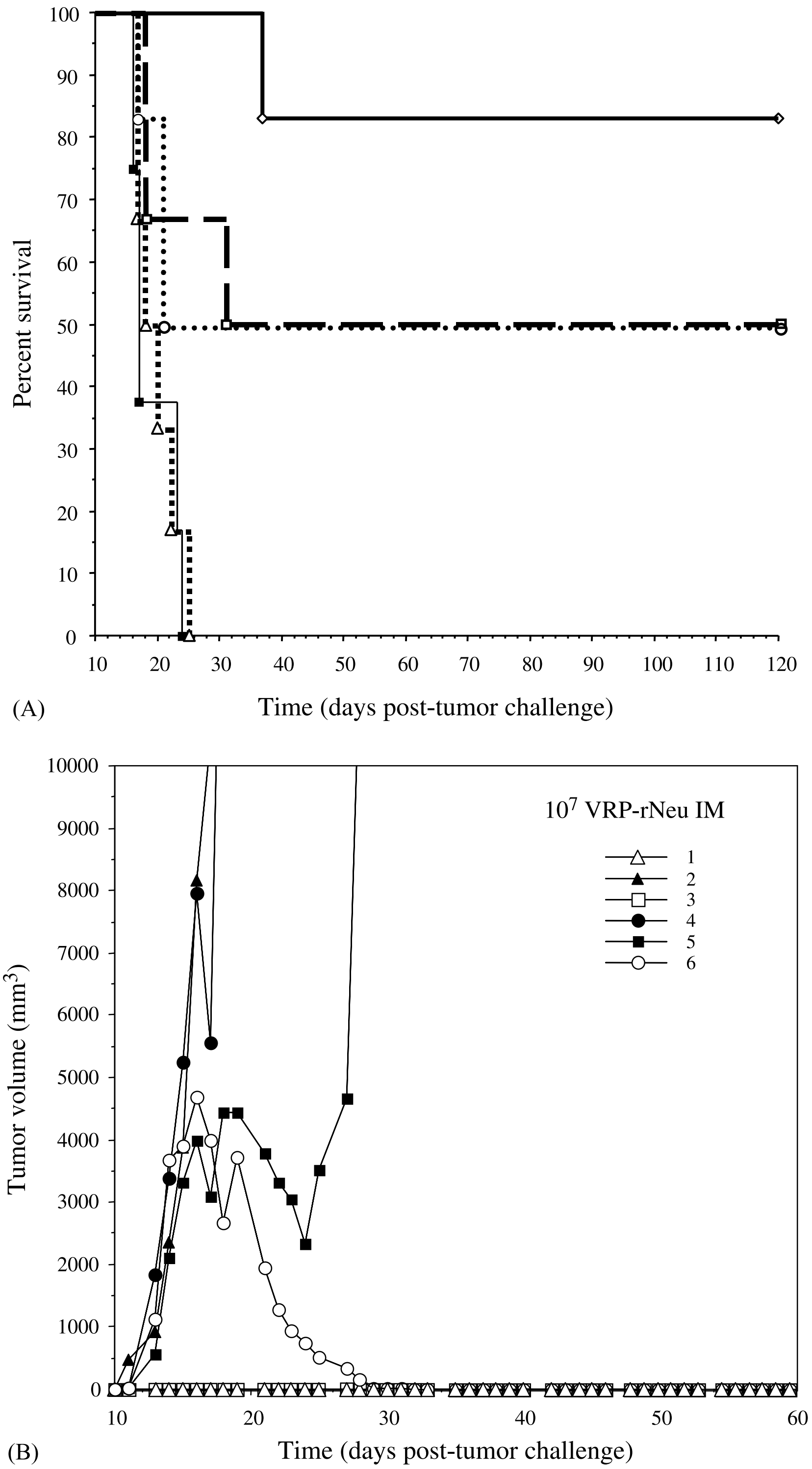

Figure 4. (A) Tumor challenge survival. Animals receiving VRP-rNeu intramuscular immunizations are depicted by the dashed line and open squares (□), VRP-rNeu SC immunizations the circle dotted line and open circles (○). Control animals, receiving VRP-HA intramuscular immunizations, irrelevant antigen for vector and specificity control, are depicted by the square doted line and open triangles (△), receiving SC immunizations of 10^6 irradiated tumor cell by the solid line and open diamonds (◊), and animals that did not receive immunization by the thin line and closed squares (■). Animals were euthanized when tumors exceeded a volume of 10,000 mm^3. (B) Individual rat tumor volumes. Each individual line represents a single animal in the cohort of VRP-rNeu intramuscular immunized animals. Time 0 equals tumor inoculation.

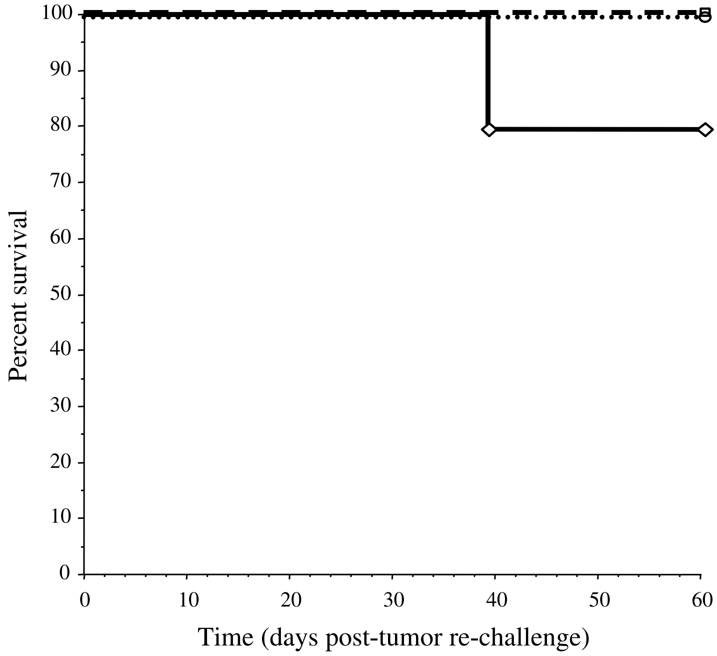

Figure 5. Tumor re-challenge survival. Survival of immunized animals receiving a second tumor challenge 140 days after the initial challenge. VRP-rNeu intramuscular route are depicted by the dashed line and open squares (□), SC immunization route by the dotted line and open circles (○), or 10^6 irradiated tumor cells by the solid line and open diamonds (◊).

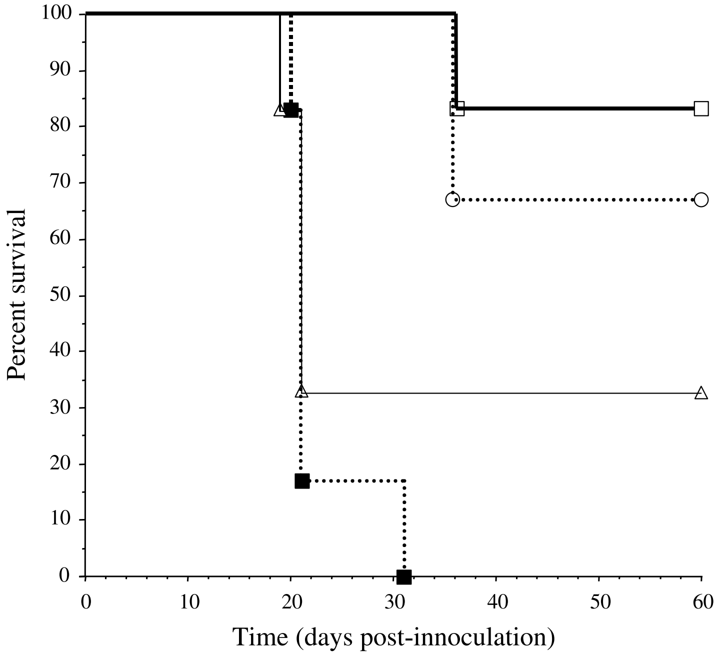

Figure 6. Winn assay. Survival of naïve animals from time of inoculation of tumor and nylon wool, T-cell enriched immune splenocytes. Splenocytes isolated from animals receiving VRP-rNeu intramuscular immunizations are depicted by the dashed line and open squares (□), VRP-rNeu SC immunizations by the circle dotted line and open circles (○), VRP-HA, the irrelevant antigen, by the square dotted line and open triangles (△) Control, and non-immunized splenocytes by the thin line and closed squares (■). Animals were euthanized when tumors exceeded a volume of 10,000 mm^3.

tumor cells, in appropriate amounts to allow for 200 μl inoculation volumes, just prior to administration to non-immunized naïve animals as described above for tumor challenges.

Results

Immunization with VRP vectors encoding the rat *neu* target antigen sequence resulted in 50% of animals being protected from tumor challenge, Figure 4. Immunization with VRP encoding the irrelevant antigen influenza hemagglutinin (HA) or sham immunization with vehicle alone resulted in no protection from tumor challenge. The survival benefit for VRP-r*Neu* immunized animals relative to control animals is statistically significant, $p = 0.004$ (two-tailed Fisher's Exact Test). The results depicted in Figure 4(a) are representative of three separate experiments that gave similar results. Interestingly, all animals receiving SC and a proportion of animals receiving IM VRP-r*Neu* immunizations developed tumors with 25–50% of these tumors permanently regressing, Figure 4(b). Overt protection from tumor challenge was observed only with the IM route of administration. There appeared to be a modest dose–response relationship over the range from 10^5 to 10^6 to 10^7 IU/immunization (data not shown).

Immunological memory was evaluated using both repeat tumor challenge and Winn assay experiments. Animals that had rejected their tumors were subjected to a second tumor challenge 140 days after the initial tumor challenge. A cohort of naïve animals was similarly challenged with tumor as a positive control and demonstrated the expected tumor development. All surviving animals previously immunized with VRP-r*Neu* were protected from a second tumor challenge while the control animals developed progressing tumors without regression, Figure 5. There were no surviving animals from the non-immunized or VRP-HA immunized cohorts. Immunologic memory elicited by the immunization procedure alone was assessed by the Winn assay. Nylon wool T cell enriched immune splenocytes purified from immunized rats were administered to naïve animals admixed with tumor at 100:1 and demonstrated significant protection, $p = 0.009$ (two-tailed Fisher's Exact Test), from tumor development relative to animals that received nylon wool T cell enriched non-immune splenocytes admixed with tumor at the same ratio, Figure 6. Nylon wool T cell enriched VRP-HA immune splenocytes protected two of six animals despite no previous demonstration of protection in challenge experiments. Flow cytometric evaluation of the nylon wool T cell enriched populations obtained from the various treatment cohorts demonstrated no significant differences in proportions of NK, CD3+, CD4+, CD8+ and MHC class II+ cells (data not shown).

Table 1. Post-immunization mean antibody titers

Immunization	Mean antibody titer	Range of antibody titers
10^7 VRP-rNeu IM	2793	1920–3840
10^7 VRP-rNeu SC	1975	640–3840
10^6 VRP-rNeu IM	2326	1280–5120
10^6 VRP-rNeu SC	988	120–2560
10^5 VRP-rNeu IM	2610	640–5120
10^5 VRP-rNeu SC	1568	480–3840
10^7 VRP-HA IM	135	<80–640
10^7 VRP-HA SC	145	<80–640
Irradiated 13762	<80	all <80
Non-immunized	<80	all <80

We evaluated serum that was obtained from animals approximately 10 days after completing the immunization sequence for antibody responses to the rat *neu* target antigen by ELISA. The IM routes of administration resulted in higher mean antibody titers at all doses, Table 1. Control animals (sham, VRP-HA, and irradiated tumor immunized) did not elicit significant anti-rat *neu* target antigen immune responses. This data is representative of the antibody titers for all three experiments in which sera was collected. All positive samples were evaluated for isotype responses. Only IgG2a or IgG2b isotypes were identified in the anti-rat *neu* target antigen reactive components. No IgG1 anti-rat *neu* antibodies were detected (data not shown). There was no correlation with antibody titer and rejection of tumor challenge.

Discussion

This series of experiments demonstrates that VEE derived VRPs are an efficient vector system for overcoming the existing level of tolerance and eliciting anti-tumor immunity to a highly conserved TAA which is also a normally expressed 'self' protein. These results were obtained in a rat tumor model that has expression of the TAA in an entirely physiologic manner in contrast to the various rat *neu* transgenic

mouse models [43–57] and using a designed TAA target antigen selected to enhance both specificity and elicited Th1/ cytotoxic T lymphocyte (CTL) immune responses. The failure of immunization with VRPs expressing an irrelevant antigen, HA, to protect animals from tumor challenge strongly supports the generation of antigen-specific anti-tumor immune responses. The long-term survival of animals that were immunized and rejected the tumor challenges, without apparent toxicity, supports the absence of significant cross-reactive autoimmune responses. The establishment of immunological memory, limited humoral responses with exclusive Th1 associated isotypes, and the capacity of nylon wool-enriched immune splenocytes to protect naïve animals from tumor challenge in the Winn assay argue for VRP-elicited Th1 biased, T lymphocyte mediated anti-tumor immune responses. Taken together these results support the further investigation of this alphavirus-derived replicon vector system for anti-tumor immunotherapy including targeting over-expressed TAAs, even those that are normal 'self' proteins.

The minimal level of protection provided by VRP-HA immune splenocytes in the Winn assay was unexpected and is not readily explained. Given the absence of tumor challenge protection and antigen reactive humoral immune responses seen with VRP-HA immunization, we believe that the effector population for this unexpected finding may reside in the innate arm of the immune system. Although there were no differences in proportion of NK or MHC II+ cells in the enriched immune splenocyte populations from the various cohorts, NK activity was not assessed and differences in activity may account for this unexpected observation. There is no reason to believe that different target antigens, HA or rat *neu*, in the VRP vector would result in different levels of NK activity. Despite this unexpected result in the Winn Assay, there is a clear antigen-specific benefit across all tumor protection, re-challenge, and Winn assays.

The breast cancer TAA HER2/*neu* is a non-mutated, member of the EGFR family [58, 59], which is over-expressed in 25–30% of human breast cancers and numerous other tumor types [60–64]. This molecule contains a number of MHC binding polypeptide T lymphocyte epitopes [65–77] and immune responses to HER2/*neu* have been described in breast cancer patients [74, 77–79]. The targeting of this molecule by the therapeutic antibody, Herceptin®, has shown clinical efficacy in a proportion of the patients that over-express HER2/*neu* [80] supporting this protein as a viable target antigen. The observation of unexpected cardiotoxicity in some patients treated with Herceptin® [80] accentuates the potential difficulties encountered when targeting a 'self' TAA.

We used a designed target antigen derived from selected portions of rat *neu*, and by extension HER2/*neu*, because we sought to both elicit immune responses biased towards Th1 and CTL effectors and to maximize specificity. Other groups have evaluated either the whole protein, extra-cellular domain, intra-cellular domain, or selected MHC binding peptides as target antigens [43–47, 67, 81–92]. The presence of large regions of highly conserved sequences in both the intra- and extra-cellular domains suggested that a designed target antigen sequence, such as ours, might avoid cross-reactive autoimmune responses while potentially allowing for processing and presentation of multiple antigenic epitopes, in the context of a broader range of MHC molecules, than with the use of MHC binding peptides. The targeting of two distinct and separate regions of HER2/*neu* by Herceptin® and this VRP based immunotherapy would suggest that these two treatments would not be antagonistic and indeed might be synergistic.

Our choice of a syngeneic rat mammary tumor model was driven by the desire to have as stringent a model as possible for evaluating antigen-specific anti-tumor immunotherapeutic strategies. The presence of a physiologically expressed, highly conserved homologue of a human TAA with its intrinsic level of tolerance more than compensated for the drawback of the restricted number of well characterized, rat specific, immunologic reagents relative to mouse systems. To date, similar studies to those described above have not been able to be performed in non-transgenic mouse models. A full-length murine cDNA homologue for human HER2/*neu* has not been demonstrated, although a number of groups have used knock-out techniques to study presumptive erbB2 function in mice [93, 94]. In contrast, the highly conserved rat homologue rat *neu*, both wild type and mutated/activated (tumorigenic) sequences, has been well characterized. Transgenic mouse models using both wild type and activated rat *neu* have been developed and have been useful in studies of anti-tumor immunotherapies including those targeting *neu* [43–57]. It is widely acknowledged that expression of transgene encoded products can result in immunologic tolerance [47] however, the expression pattern is not always consistent with normal physiology. Additionally, the mammary tumor cell line we selected expresses relatively low levels

of MHC class I on the cell surface, recapitulating findings in human breast cancers [95], and modestly over-expresses rat *neu* both of which add rigor to the model. Thus, we believe that rat *neu* and rat mammary tumor models are more clinically relevant for evaluating both the capacity to elicit anti-tumor, antigen-specific immune responses and any untoward autoimmune phenomena.

Several characteristics of VEE derived VRPs appear to contribute to the efficiency of these vector systems, including (1) the very high level of heterologous gene expression, (2) their specific cell and tissue tropisms, and (3) the vector-associated cytopathic effect. The life cycle of alpha viruses and alphaviral-derived vectors occur entirely within the cytoplasm that obviates transport of genetic material across the nuclear membrane as a concern and removes the potential for chromosomal integration or adventitial splicing of engineered coding sequences. The genomic positive strand RNA gives rise to amplified subgenomic RNAs that are then directly translated for the synthesis of the encoded heterologous protein resulting in very high levels of viral encoded protein synthesis, up to 25% of total cellular protein [41, 96], providing abundant albeit transient expression of heterologous proteins. The expression of the heterologous protein is intracellular and thus, available to the endogenous antigen processing and presentation pathway. The host tissue receptivity for VEE and derived replicons includes lymphoid and neuronal cells, and specific targeting to DC has been described [15, 16]. The cytopathic effect, believed to result primarily in apoptotic cell death [30, 97–114], can result in uptake of heterologous protein contained in the cellular debris via the extrinsic antigen-processing pathway for presentation on MHC class II and in the case of DCs also ‘cross-priming’ into the MHC class I pathway. Furthermore, the associated cytopathic effect may yield pro-inflammatory signals and be implicated in enhanced expression and loading of host heat shock proteins with peptides from expressed heterologous polypeptides.

Alphavirus replicon vectors, derived from VEE virus, have recently been used for targeting xenoantigens in two other models of anti-tumor immunotherapy. The full length prostate specific membrane antigen (PSMA) coding sequence has been used as the heterologous protein in one system [115, 116]. Mice immunized with VRPs designed to express PSMA developed both antibody and cellular immune responses reflective of a Th1 bias as characterized by cytokine production and murine antibody isotype. Velders et al. evaluated human papilloma virus (HPV) E7 as a target antigen in a VRP immunization strategy [27] using a mouse model consisting of C57BL/6 mice and the syngeneic C3 tumor cell line expressing HPV-16 E7. This VRP immunization regimen provided 100% protection against the tumor challenge and was associated with increased E7 specific CTL responses, E7 peptide tetramer staining of CD8+ T cells, and IFNγ ELISPOT responses. Evaluation of the E7-VRP immunization strategy in a 1-week palpable tumor nodule treatment model resulted in regression of tumor in 67% of the animals at 60 days. This vector system has also been extensively evaluated in prophylactic immunization strategies for various other infectious diseases which have yielded important mechanistic and toxicity data [32–41].

The data contained within this report, along with that reported by the groups targeting HPV E7 and PSMA, suggest that the VEE VRP vector system is a very potent anti-tumor immunotherapeutic vector system. The absence of toxicity in immunized animals capable of rejecting a tumor challenge, in our report, suggests that non-mutated ‘self’ proteins can be targeted by the strategic selection of regions to be targeted. Using this vector system and the designed target antigen, the pre-existing level of tolerance can be overcome and effective anti-tumor immunity can be elicited to an over-expressed ‘self’ TAA without apparent development of clinically relevant cross-reactive autoimmunity. Studies are underway to more extensively characterize the VRP elicited anti-tumor immune response and this data will facilitate design of immunologic monitoring strategies for potential clinical trials. The capacity to mount an effective immune response to a tumor that moderately over-expresses the non-mutated TAA *neu* suggests that this strategy may be more broadly applicable than existing antibody therapies that require 3+ immunohistochemical over-expression or gene amplification. This strategy, including the designed target antigen, could be readily translated into human clinical trials due to the exceptional homology between rat *neu* and HER2/*neu*, and could be a valuable addition to therapeutic armamentarium for patients with breast cancer.

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